

AperTO - Archivio Istituzionale Open Access dell'Università di Torino

Effects of the rare elements lanthanum and cerium on the growth of colorectal and hepatic cancer cell lines

This is a pre print version of the following article:

Original Citation:

Availability:

This version is available <http://hdl.handle.net/2318/1690018> since 2019-02-05T11:14:09Z

Published version:

DOI:10.1016/j.tiv.2017.09.024

Terms of use:

Open Access

Anyone can freely access the full text of works made available as "Open Access". Works made available under a Creative Commons license can be used according to the terms and conditions of said license. Use of all other works requires consent of the right holder (author or publisher) if not exempted from copyright protection by the applicable law.

(Article begins on next page)

1 **Effects of the rare elements lanthanum and cerium on the growth of colorectal and hepatic**
2 **cancer cell lines**

3 Alessandro Benedetto^{1*}, Claudia Bocca², Paola Brizio¹, Stefania Cannito², Maria Cesarina Abete¹,
4 Stefania Squadrone¹

5 ¹ Istituto Zooprofilattico Piemonte Liguria Valle d'Aosta, Torino, Italy

6 ² Università di Torino, Dipartimento di Scienze Cliniche e Biologiche Unità di Patologia Generale,
7 Torino, Italy

8
9 *Corresponding author, e- mail address: alessandro.benedetto@izsto.it

10 **Abstract:**

11 Human HT-29 and HepG2 cell lines were employed to test the effects of increasing concentrations
12 of the REEs cerium (Ce) and lanthanum (La), alone or in combination. Effects on cell proliferation
13 were measured using MTT colorimetric assay and confirmed by RealTime-Glo™ MT Cell Viability
14 Assay and the evaluation of the proliferation marker PCNA, while cell mortality and type of cell
15 death was determined by Annexin V binding to phosphatidyl serine at the cell surface of apoptotic
16 cells using flow cytometry. The modulation of 84 genes, targeting pathways of oxidative stress was
17 also studied using arrays based on RT-PCR. Major alterations in selected genes from basal
18 expression levels of respective control groups were found in the 600 µM cerium exposed cells
19 (48h). In HepG2 cells 51 out of 84 genes resulted significantly up/down regulated, while in HT-29
20 cells only 16 genes resulted significantly up/down regulated. The dosage of REEs seems to
21 represent the pivotal factor for switching the biological effects from down to up-regulation of cell
22 growth; thus, the low concentrations promote cell survival and proliferation, but when
23 concentrations increased, REEs exert anti-proliferative and cytostatic/cytotoxic effects.
24 The molecular mechanisms underlying these effects are still not well defined and further analysis of
25 the mechanisms which result in inhibition or induction of cell proliferation is crucially important.
26

27 **Keywords:** lanthanum; cerium; human cell lines; toxicity; cell growth.
28
29
30
31
32
33
34
35
36

1. Introduction

Lanthanum (La) and cerium (Ce) belong to the rare earth elements (REEs) that have many uses in industries throughout the world. As a group, rare earths constitute the fifteenth most abundant component of the earth's crust; cerium occurs more often in the earth's crust than lead, molybdenum or arsenic, and lanthanum occurs in similar quantities as lead, then they are not rare at all (Redling, 2006). In the last decade, REE has become one of the common xenobiotics in our surroundings (Zhao et al., 2004) as it is widely used in industry, stockbreeding and medicine, especially as trace fertilizers in agriculture, thus they can be concentrated by food chain.

China contains the largest mineral deposits of REEs in the world (Drew et al., 1990; Yang and A., 2006) and the application of rare earth elements as feed additives for livestock has been practiced in China for decades: the elements lanthanum, cerium and others are used as feed additives in animal production (Redling, 2006). Numerous reports in the Chinese literature described that a small amount of these REE mixtures in the diet can increase the body weight gain of pigs, cattle, sheep and chicken and it was reported that they increase milk and egg production (He and Xia, 1998; Rambeck and Wehr, 2000; Zhu et al., 1994).

Rare earth elements have several properties that make them attractive alternatives to antibiotics as growth promoter. Generally, absorption of orally applied rare earths is low with more than 95% being recovered in the feces of animals (Redling, 2006). As a result, the chances of residues being present in meat are low with studies reporting no higher levels of rare earth elements in the muscle tissue of supplemented animals than those fed commercial diets (Redling, 2006).

If only a very small amount of REE could be absorbed into body when they were supplemented orally (He and Rambeck, 2000), however, such small amount of REE in animal body may have effect on the metabolism through influence the hormones such as triiodothyronine (He et al., 2003) or growth hormone (Wang and Xu, 2003). Results indicate that the supplementation of REE to the media may affect adipogenesis and lipogenesis rates of 3T3-L1 mouse cells and that the effect may depend upon the dose or type of REE applied.

About a dozen of studies, especially in pigs, were performed under western animal production conditions (Rambeck et al., 2004). Most of these studies in piglets and in fattening pigs provided significant data, indicating that REE imported from China can improve weight gain and feed conversion (Rambeck et al., 2004). In addition, there have been no reports of the development of bacterial resistance in treated animals (Redling, 2006).

However, from October 2004 only that feed additives that passed a renewed European Food Safety Authority (EFSA) procedure can be put on the market in the European Union. Under these new rules feed additives will be categorized as technological additives, zoo technical additives and coccidiostats and histomostats (Khan, 2004). If REE have the character of trace elements, they belong to the nutritional additives, if they enhance the digestibility or stabilize the gut flora, they are zoo technical additives. In Switzerland, REE obtained a temporary registration under the trade name “Lancer” to be supplemented like other essential trace elements to the feed of piglets and pigs at a concentration between 150 and 300 mg REE per kg feed.

The European Food Safety Authority recently evaluated the safety and efficacy of Lancer as feed additive for weaned piglets (EFSA, 2013). Lancer is a feed additive mainly consisting of two rare earth elements, lanthanum and cerium, and although one study (Von Rosenberg et al., 2013) suggests that La and Ce are not deposited in tissues in piglets, this is apparently not consistent with data found in other species. e.g. cattle (Schwabe et al., 2011). Consequently, EFSA did not conclude that there is no potential for consumer exposure; in addition, in the absence of an established NOAEL (no observed adverse effect level) for the target species, EFSA was unable to relate any possible exposure to evidence of a safe dose.

In view of the increasing requests of application of lanthanides for improving animal growth, which may increase the possibility of human exposure, it is becoming necessary to obtain in-depth information on their environmental toxicity in impact on humans (Dai et al., 2002; Xue et al., 2009). It is well known that the biological effect spectrum of REE is wide and the dose-response relationship is complicated. Studies in rats have shown that REE could induce chromosome

89 damage of blood lymphocytes (Xu et al., 2000) and liver damage (Nakamura et al., 1997) depress
90 learning and memory (Li et al., 2000) increase or suppress cell-mediated immunity of the spleen
91 (Liu et al., 2000), change the expression levels of some kinds of genes (Zhao et al., 2004).
92 The safety evaluation of REE effects in animals and humans is difficult, and it is necessary to find
93 sensitive biomarkers, utilizing different techniques to acquire a deeper understanding of their
94 mechanism.
95 In this perspective, we explored the effects of different dosages of lanthanum and cerium on cell
96 viability and proliferation in two human cancer cells lines, human colorectal (HT-29) and
97 hepatocellular (HepG2), as well as the study of the modulation of 84 genes targeting pathways of
98 oxidative stress in humans. In our knowledge, there are no previous studies considering all these
99 aspects to elucidate the biological and molecular mechanism of REE toxicity in humans.

100

101 **2. Material and Methods**

102 **2.1. Chemicals**

103 Cerium (III) chloride heptahydrate, 99.9% purity grade and Lanthanum Chloride heptahydrate,
104 99.999% purity grade were purchased from Sigma–Aldrich (Milan, Italy). All other chemicals and
105 reagents used were of analytical grade.

106

107 **2.2. Cell culture**

108 Human hepatoma HepG2 and Colon adenocarcinoma HT-29 cell lines were respectively cultured in
109 DMEM and McCoy's 5A medium containing 10% fetal bovine serum (FBS) (Gibco BRL, Grand
110 Island, USA), penicillin, 100 µg/mL streptomycin and amphotericin B (Sigma Chemical Co., St
111 Luis, MO) at 37 °C in a 5% CO₂. Different batches of described media were prepared adding
112 lanthanum and cerium, alone or in combination, in concentrations ranging from 0.1 µM to 10 mM
113 and then filtered with filtropur 0.2 µm (SARSTEDT). Different biological replicates for each
114 treatment condition, defined by exposure time and compound concentration, was then prepared for

the following analysis steps: quantification of effective exposure doses by ICP-MS, cell mortality and cell cycle progression by flow-cytometry, cell proliferation by MTT assay and gene expression profiling by quantitative RT-PCR. Negative control cell cultures for each time point and for both cell types were included in the experiment.

2.3. ICP-MS

The concentrations of all lanthanides solutions was checked by ICP-MS, in order to take in consideration possible effects of filtration and precipitation of studied compounds in medium used for cultured cells. Determination of Ce and La was performed after wet digestion with acids and oxidants (HNO_3 and H_2O_2) of the highest quality grade (Suprapure). About 0.5 g of material were subjected to microwave digestion (microwave oven ETHOS 1 from Milestone S.r.l., Sorisole (BG), Italy) with 7 ml of HNO_3 (70% v/v) and 1.5 ml of H_2O_2 (30% v/v). Samples were then bring at the final weight of 50 g with ultrapure water (Arium611VF system from Sartorius Stedim Italy S.p.A., Antella - Bagno a Ripoli, (FI), Italy). Ce and La determination was performed with ICP-MS (XseriesII, Thermo Scientific, Bremen, Germany) after daily optimization of instrumental parameters and external standard calibration curve; terbium was used as internal standard.

2.4. MTT assay

MTT assays were performed to determine cell proliferation (Mosmann, 1983). HepG2 and HT-29 cells were treated in 96 well plates with fixed concentrations of Cerium and Lanthanum (Table 1). After incubating cells for 24 h, 48 h and 72 h with lanthanum/cerium single and mixed compounds, cell proliferation was estimated by the MTT assay as follows: 5 mg mL^{-1} of MTT reagent (Sigma–Aldrich) was added and incubated for 3 h at 37 °C in humidified atmosphere (5% CO_2). After

138 incubation, the media was removed and 100 μ L of DMSO was added to each well to dissolve the
139 formazan (the metabolic product from MTT). Then, the absorbance at 590 nm was measured in a
140 microplate reader (Spectramax, Gemini, EM). Results are expressed as percentage of means \pm SD
141 (Standard Deviation) of 3 experiments, each conducted in 6 replicates, calculated with respect to
142 control considered as 100%.

143

144 *Western blotting*

145 HepG2 and HT-29 cells were plated in 6 well plates (10^5 cells/well) and treated for 24 and 72 h,
146 respectively, with lanthanum/cerium single and mixed compounds. Total cell lysates, obtained as
147 previously described, (Cannito et al., 2008) were subjected to sodium dodecyl sulfate-
148 polyacrylamide gel-electrophoresis on 12% acrylamide gels, incubated with desired primary
149 antibodies (PCNA sc-25280 ; primary antibody dilution was in agreement with the manufacturer's
150 instructions), then with peroxidase-conjugated anti-mouse immunoglobulins in Tris-buffered saline-
151 Tween containing 2% (w/v) non-fat dry milk and finally developed with the ECL reagents
152 according to manufacturer's instructions. Sample loading was evaluated by reblotting the same
153 membrane with β -actin antibody.

154

155 *2.5. Flow Cytometry*

156 Cell mortality and type of cell death was determined by Annexin V binding to phosphatidyl serine
157 at the cell surface of apoptotic cells using flow cytometry.

158 Detection of apoptosis was carried out using annexin V-FTC staining according to the
159 manufacturer's instructions (Immunostep). HT-29 and HepG2 cells (2×10^5 well) were seeded in 6-
160 well plates in 2 ml serum-free medium and incubated for 24 h; the cells were then treated with

lanthanum and cerium at the indicated concentration for 24 and 48h. A negative control was prepared by untreated cells. Following incubation, floating cells were collected and adherent cells were digested by trypsin without EDTA to detach them. The cells were washed twice in temperate phosphate-buffered saline (PBS) and resuspended in 1X Annexin-binding buffer at a concentration of 1×10^6 cells/ml. 5 μ l of Annexin V-FITC and 5 μ l of propidium iodide were added to each 100 μ l of cell suspension. The cells were incubated at room temperature for 15 min in the dark, and then 400 μ l of 1x Annexin binding buffer was added. The samples were analyzed by flow cytometer (BD Accuri C6); 20,000 events were collected per sample. Results are provided as mean \pm SD of 2 experiments conducted in triplicates and the rela.

170

Cell viability was also evaluated by using RealTime-Glo™ MT Cell Viability Assay (Promega Italia, MI). HepG2 cells and HT-29 cells were plated into 96 well opaque-walled assay plates (10×10^4 cells/well), treated with lanthanum/cerium single and mixed compounds and incubate for 24 and 72h. After treatment, the cells were incubated for 10 min in the cell culture incubator with a RealTime-Glo™ reagent according to the manufacturer's protocol. Luminescence was then measured on a Glomax Multi Detection System Promega with an integration time of 0.5 second per well. The luminescent signal correlates with the number of metabolically active cells. Results are provided as mean of luminescence values (RLU) \pm SD of 2 experiments conducted in 6 replicates.

179

For cell cycle analysis, HepG2 and HT-29 cells (2×10^5 well) were seeded in 6-well plates in 2 ml serum-free medium and incubated for 24 h; the cells were then treated with lanthanum and cerium for 24, 48 and 72h. A negative control was prepared by untreated cells. Following incubation, floating cells were collected and adherent cells were digested by trypsin without EDTA to detach them. The cells (1×10^6 /ml) were then centrifuged at 3000 rpm for 10 min at 4°C. The pellets obtained were resuspended in 70% ethanol, incubated for 1h at 4°C and then centrifuged at 1000 rpm for 10 min at room temperature. The cells were resuspended in 600 μ l of PBS, incubated with

187 100 µl RNAase (4 mg/ml) for 30 min at room temperature and then with propidium iodide (1.8
188 mg/ml) for 10 min at room temperature. The samples were analyzed by flow cytometer (BD Accuri
189 C6). Results are provided as mean \pm SD of 2 experiments conducted in triplicates. Sub G0-G1
190 phase identify events not included in the normal cell cycle phase distribution but observed by flow
191 cytometry.

192

193

194 2.6. Gene expression profiling using oxidative stress qPCR-based arrays

195 The modulation of 84 genes, targeting pathways of oxidative stress in humans was studied using
196 arrays based on quantitative RT-PCR (qPCR)-analysis (RT² Profiler™; SA Biosciences, Milan,
197 Italy), performed according to the manufacturer's protocol. The array used in this study (Ref.
198 PAHS-065ZC) consisted of a 96-well plate containing primers optimized for amplification of the
199 targeted genes, plus five housekeeping genes and seven internal controls, including evaluation of
200 reverse transcription, qPCR and gDNA removal efficiencies, as described in previous work (Rouimi
201 et al., 2012; Shah et al., 2016). RNA extraction was performed on each sample (containing 7×10^5 –
202 2×10^6 cells) by miRNeasy Plus mini kit (Qiagen, Milan, Italy). RNA concentration was estimated
203 by Q-Bit fluorimetric assay (Thermo Scientific, Wilmington, DE, USA), while RNA integrity and
204 quality were controlled using an Agilent 2100 Bioanalyzer. 0.5 µg of total RNA from each
205 individual was converted into cDNA using the RT² First Strand Kit (SA Biosciences/QIAGEN).
206 RT²Real-Time SYBR Green/ROX PCR Master Mix was added to the cDNA and an equal volume
207 of 25 µl was distributed to each of the 96 wells of the array. Amplification was performed on an
208 ABI StepOne Plus thermocycler with an initial 10-min hot start step at 95 °C followed by 40 cycles
209 at 95 °C for 15 s and 60 °C for 1 min. A dissociation curve was run after 40 cycles to verify single
210 product formation.

211 Cycle thresholds from the real-time PCR was exported to an excel file and analyzed using web-
212 based PCR Array Data Analysis Software available at

213 www.SABiosciences.com/pcrarraydataanalysis.php, allowing determination of normalized
214 average C_t values, paired t test p -value and fold change calculations, based on $2^{-\Delta\Delta C_t}$ method
215 (Livak and Schmittgen, 2001). Experiment were performed in triplicate for each treatment. Genes
216 differentially expressed were retained when the P -value was < 0.05 and fold change > 2 following
217 the different treatments.

218

219 **Statistical analysis**

220 Statistical analysis was performed by Student's t -test or ANOVA for analysis of variance when
221 appropriate ($p < 0.05$ was considered significant).

222

223 **3. Results**

224 **3.1 Effects on cell proliferation**

225 Effects of increasing concentrations of cerium (Ce) and lanthanum (La), alone or in combination, on
226 human colorectal (HT-29) and hepatocellular (HepG2) cancer cells were measured using MTT
227 colorimetric assay. The results obtained for HepG2 cells are summarized in Table 1. The highest
228 cell proliferation rate was observed in the group exposed for 48 and 72 hours to 25 μM of La+Ce
229 when compared with control cells group, while 250 μM of La+Ce halved cell proliferation at all
230 time point. Considering Ce and La separately, proliferation trends in HepG2 cells exposed to
231 lanthanum were described in all groups stimulated with doses below 70 μM for 48 and 72 h, while
232 at concentration of 180 μM , La inhibited cell proliferation. Ce stimulation of HepG2 cells revealed
233 in general lower proliferation trends and concentration of 300 μM halved proliferation rate after 48
234 h exposure.

235 In HT-29 cell line, the supplementation of low concentration of lanthanides as a mixture or as a
236 single compound (La 20 μM /24h group and La+Ce 25 μM /72h group respectively) increased the
237 relative ratio of absorbance detected by MTT assay, indicating a considerable growth-promoting
238 effect (Table 1). The inhibitory effects of Ce appeared only at concentration tested of 300 μM and

239 250 μ M of La+Ce), while La as single compound induced a significant inhibition of cell growth at
240 370 μ M at all time considered. In addition, after 48-72h exposure with 300 μ M Ce, the initial
241 inhibition of growth resulted overcome and cell proliferation restarted, reaching levels near to
242 untreated controls. The tendency observed by using MTT assay was also confirmed by the analysis
243 with RealTime-Glo™ MT Cell Viability Assay (Figure 1).

244 The influence of Lanthanides on cell proliferation was also evidenced in Figure 2 in which the
245 expression of proliferation marker PCNA (Proliferating Cell Nuclear Antigen) was reported.
246 According to previous results, in HepG2 cells PCNA expression was augmented at low
247 concentration of La (70 μ M) and Ce (120 μ M) as well as at 25 μ M of La+Ce, while it was
248 reduced at high concentrations.. The same trend was detected in HT-29 cells: 20 μ M La and 25 μ M
249 of La+Ce induced PCNA expression, while high dosed of La and Ce as mixture or as a single
250 compound, exerted inhibitory activity.

251

252 ***3.2 Effects on cell cycle distribution and apoptosis induction***

253 HepG2 and HT-29 cells were treated for 24, 48 and 72 h with tested compounds at a range of
254 concentration able to interfere with cell proliferation. Since the inhibition of cell growth may result
255 from either cell cycle arrest or cell death, we performed cell cycle analysis by flow cytometry after
256 treatment with various dosages of La and Ce (Table 2). Annexin V flow cytometry analysis was
257 applied to quantify the apoptotic profile (Table 3). The results obtained in HepG2 cells showed a
258 significant presence of apoptotic cells after 48h exposure to both La (180 and 370 μ M) and Ce (300
259 and 600 μ M), with the highest level of apoptosis of about 29%. In this cell-line, lanthanides did not
260 significantly alter cell cycle distribution, as shown in Table 2.

261 On the contrary, in HT-29 cells none of the tested compounds induced apoptotic cell death (data not
262 shown). According to viability data, cell cycle analysis showed that increasing dosage of both La
263 and Ce caused a gradually and significative reduction of cells in S phase, accompanied with a
264 simultaneous increase of the population in G0-G1 phase (Table 2).

265

266 *3.3 Gene expression profiling*

267 Gene expression analysis on HepG2 and HT-29 cell lines, stimulated with single and combined
268 Lanthanum and Cerium doses, was limited to 48h time point, where a significant reduction in
269 proliferation rates, established by MTT analysis, was recorded (as shown in Table 1).

270 From analysis pipeline applied to HT-29 expression profiles in treated and untreated control cell
271 lines 26 out of 84 genes resulted significantly up/down regulated. Gene list and fold changes were
272 reported in Figure 1A and 1B. Supplemental material with fold regulation of each single gene is
273 also provided. The major alterations in selected genes from basal expression levels recorded in
274 respective control groups were found in the 600 μ M cerium exposed (48h).

275 In HepG2 cell-lines, the expression analysis was limited to six groups at 48h, chosen because of
276 previous proliferation tests. Gene list and fold changes were reported in Figure 2A and 2B; 47 out
277 of 84 genes resulted significantly up/down regulated. Like for HT-29, also in HepG2 cell lines the
278 main alterations in considered gene panel were found in higher cerium exposed replicates.

279

280 **4. Discussion**

281 The analysis of the effects of lanthanides on cell proliferation revealed that the concentration used
282 represents the pivotal factor for switching the biological effects of lanthanides from toxicity to
283 activity.

284 According to Chen and coauthors (Chen et al., 2000) the effects of lanthanides on cell growth is bi-
285 directional, depending on the concentration used and cell lines. In our study, the results obtained
286 from MTT and RealTime-Glo™ MT Cell Viability Assay evidence how La produces growth-
287 promoting effects in HT-29 cells in a range of relative low concentrations (20 μ M). Ce does not
288 seem to promote HT-29 growth, since the main effect of Ce is indeed inhibition of cell proliferation,
289 higher than that observed for La, being present within 24 h at high exposure concentrations.

290 Interestingly, after prolonged exposure of cells to high concentration (300 μ M) of Ce, the initial
291 inhibition of growth resulted overcome and cell proliferation restarts, reaching levels near to
292 untreated controls. This evidence seems to suggest a reversible block of cell proliferation, consistent
293 with cell cycle analysis that revealed the presence of a percentage of cells in G2-M phase similar to
294 that detected in control cells. In this regard, both Ce and La block HT-29 cells in the G0-G1 phase,
295 consistently with the inhibition of cell proliferation, while at low doses increase the percentage of
296 cells in S and G2-M phases, according to promoting effects.

297 Recently, La was found to inhibit the proliferation and promote the apoptosis in some cancer cells
298 (Wang et al., 2016; Yu et al., 2015b). The results obtained in our study seem to suggest that in HT-
299 29 cells the inhibition of proliferation by lanthanides is related to a cytostatic rather than a cytotoxic
300 effect, without apoptotic induction.

301 The combination of Ce and La causes a boosted promoting effect on cell proliferation at low dose
302 (25 μ), while at high dose (250 μ M) La+Ce exert a more significative inhibitory effect.

303 In HepG2 cells, La promoted cell growth in a larger range of concentrations especially on 48 h and
304 72 h groups, while the inhibition of cell proliferation needed doses of 180 μ M to be recorded. Ce
305 exposure of HepG2 lines does not provide any growth-promoting effects, but at the highest
306 concentration tested it induced the inhibition of cell growth at all time point. At these doses, the
307 inhibitory effect of both lanthanides seems to be related to apoptotic cell death induction, as
308 revealed by annexin V test.

309 Finally, cell exposure to La and Ce in combination produces an increase of the proliferation rate
310 after 48-72 h incubation at the lowest tested concentrations, while only prolonged incubation with
311 250 μ M lanthanides is related to a significant increase of cell inhibition.

312 In both cell lines, the tendency of growth promoting and inhibiting effects exerted by Lanthanides
313 was confirmed by the evaluation of the expression of Proliferating Cell Nuclear Antigen, an
314 auxiliary protein of DNA polymerase delta involved in the control of DNA replication (Wang,

2014). According to proliferation data, our results evidenced that the expression levels of PCNA was increased at low doses of lanthanides and down-regulated at high doses.

Studies on toxicities of REE elements by different intake pathways showed that the liver was the target organ of REE element toxicity in mice (Arvela et al., 1991; Chen et al., 2000), and then we choose the hepatocellular (HepG2) cancer cell line to provide a unique and powerful experimental system to study differential gene expression in humans. In addition, since no data exists on the effects of lanthanides in colon cancer cells, the analysis was also performed on human colorectal (HT-29) cancer cell line.

Among cerium treated HT-29 cell lines, the Heme oxygenase 1 (HMOX1), Thioredoxin reductase 1 (TXRND1), Glutamate-cysteine ligase (GCLC), Myoglobin (MB) and Ferritin (FHT1) were the most interesting upregulated genes after 48 h exposure to Ce 600 μ M. As previously reported (Valdes et al., 2013), the corresponding enzymes/proteins usually exert early cytoprotective effects against different toxic elements and molecules, promoting the phase II excretion and/or redox inactivation of such toxic compounds to reduce injure of biomolecules such as nucleic acids, proteins and lipids (Martin et al., 2004). Another gene significantly upregulated in HT-29 after Ce exposure was Glutathione peroxidase-3 (GPX3), coding for a selenoprotein that is the extracellular member of a family of plasma glutathione peroxidases (GPXs). In a recent study performed on different REEs in hepatocyte nuclei, superoxide dismutase (SOD) and catalase (CAT) activities decreased after cerium exposure while GPXs activities, GSH and malondialdehyde (MDA) levels increased compared to the control group (Huang et al., 2011). The authors found opposite effects in hepatocyte mitochondria, where GPXs activities and GSH levels were significantly decreased, underlining how REEs could selectively accumulate in different cellular compartments inducing oxidative damage.

SQSTM1 encoding the p62 protein (sequestosoma 1), which plays an important role in the process of autophagy (Salminen et al., 2012), a process that provides to protect the cell by removing protein deposits altered and was found mutated in neurodegenerative diseases (Alzheimer's disease). In our

study, this protein was found to be up regulated in all treated groups and being involved in the elimination of toxic substances could indicate the necessity that the cells have to detoxify by the presence of toxic levels of cerium.

Considering the downregulated genes in HT29 cell lines, the major alteration was recorded in 48 h Cerium 600 μ M group: the expression of Forkhead box M1 (FOXO1), Methionine sulfoxide reductase A (MSRA), Prostaglandin-endoperoxide synthase 2 (PTGS2), Cytoglobin (CYGB) and Myeloperoxidase (MPO) resulted strongly inhibited. Among these genes only CYGB was constitutively inhibited also in all groups treated with single cerium and lanthanum after 48 hour exposure, suggesting a common effect of different single REE elements on CYGB expression in HT-29 cell lines. CYGB is a known oxidative stress-responsive gene, due to its capacity to bind ROS and nitric-oxid (Liu et al., 2013). Hypoxic upregulation of CYGB as well as its altered expression in various human cancers suggest a possible role of this globin in tumor cell response under low oxygen tension (Chakraborty et al., 2014). Conversely, CYGB inhibition was not recorded with combined stimulation with La and Ce. Moreover, the same target, analyzed in HEPG2 cell lines, shown a completely different behaviour, with a strong upregulation within 48 hours exposure to both La and Ce (Figure 2).

Indeed a different range of effects was recorded for HEPG2 cells when compared to HT-29. In HEPG2 cell lines lower amounts of single REEs (70 μ M of La, 120 μ M of Ce respectively) induced few alterations in analyzed genes when compared with higher doses (180 μ M of La, 600 μ M of Ce). Interestingly in HEPG2 cell exposed to Ce 120 μ M only inhibition effects on 19 target was recorded (Figure 2), with no upregulation of any of 84 considered genes. However, when Ce concentration was 600 μ M, an upregulation of 7 marker was observed, with the highest expression levels of lactoperoxidase - LPO (up to 25 fold when compared with untreated control group), as well as chemokine (C-C motif) ligand 5 (CCL5), myeloperoxidase (MPO) and glutamate-cysteine ligase (GCLC). This reproduce a known pathway of oxidative stress response in HEPG2 cells (Pyo et al., 2016) based both on the production of antioxidant enzymes and phase II enzymes and on the

367 excretion of inflammatory chemokines like CCL5 that are critical for macrophage recruitment to the
368 liver tissues (Barashi et al., 2013).

369 Another upregulated marker of REE exposure, not only in HT29 but also in HEPG2 (Figure 1 and
370 2), is Myoglobin (MB). MB is the oxygen carrier of skeletal and heart muscle but it is known to
371 contribute in nitric oxide homeostasis and in reducing oxidative stress by scavenging hydrogen
372 peroxides increased by hypoxia and following re-oxygenation after hypoxic events (Helbo et al.,
373 2013). Recent studies on hypoxia-regulated expression of the MB gene in different cancer cell lines
374 and breast cancer tissues revealed how this globin could exert a cancer-suppressive role by
375 impairing mitochondrial activity (Bicker et al., 2014). The ectopic tumor-suppressor function of
376 MB found in hypoxia-responsive cancer cell lines studied by Bicker and coauthors, may partly
377 explain the effects seen on proliferation decrease of both HEPG2 and HT29 cell lines when exposed
378 to higher concentration of REE (Table 1).

379 Among considered glutathione peroxidases (GPXs), previous studies shown that both over-
380 expression (Guariniello et al., 2015) and catalytic activities (Guerriero et al., 2015) of GPX4 and
381 GPX7 resulted strictly related to human hepatocellular carcinoma progression. The down regulation
382 of both GPX4 and GPX7 on HEPG2 cell lines after 48h exposure to higher cerium concentration
383 (Figure 2) confirmed the anti-tumoral effects of this rare element through inhibition of selenium-
384 containing peroxidases. The radically different behaviour of GPXs expression in HT-29 (GPX4
385 strongly up-regulated by all REE exposure protocols) suggest important differences between the
386 two cell lines considered in current study.

387 Among downregulated markers in HEPG2 cell lines following single La or combined La and Ce
388 stimulation the most inhibited gene was PXDN, respectively from 16 to 22 fold lower in HEPG2
389 exposed to La 70 and 180 μ M, and from 21 to 20 fold lower when exposed to combined La+Ce 100
390 μ M and La+Ce 250 μ M (Figure 3).

391 On the contrary, in all Ce only exposed groups no alteration of PXDN was recorded. PXDN
392 (peroxidasin) is a secreted heme-containing peroxidase primary involved in the extracellular matrix

393 formation, but it shares similar function of Phagocyte-derived myeloperoxidase (MPO) and other
394 secreted peroxidases (Soudi et al., 2015), representing a versatile class of enzymes necessary to
395 manage both physiological and pathogenic functions, linked to immune and oxidative stress
396 response. In our experimentation the radically different behaviour of heme-peroxidases like
397 MPO/LPO (highly induced) and PXDN (strongly inhibited) suggest a controversial implication of
398 peroxidases in response to lanthanides induced oxidative stress, confirming how peroxidase
399 families, also if characterized by high conserved catalytic domain, have been described to evolve
400 diverging during evolution (Li et al., 2012).

401 Regarding mitochondrial genes included in the study major alteration were recorded in expression
402 of UCP2, the Uncoupling protein 2 (mitochondrial, proton carrier), MPV17 (mitochondrial inner
403 membrane protein) and mitochondrial isoform of super oxide dismutase (SOD2).

404 UCP2 is known to suppress mitochondrial reactive oxygen species (ROS) generation, mitigating
405 oxidative stress-induced apoptosis. Recent studies in different hepatocellular carcinoma cells
406 (HCC), including HEPG2, show a basal high expression of UCP2, related to chemotherapeutic
407 agents' resistance (Valle et al., 2010). The specific knockdown of UCP2 expression by targeted
408 siRNA enhanced the Gemcitabine induced inhibition of HCC growth (Yu et al., 2015a). The similar
409 inhibition of UCP2 promoted by REE exposure in HEPG2 found in current study confirm the key-
410 role of this mitochondrial proton carrier in HCC proliferation. Conversely, in REE treated HT29
411 lines UCP2 expression did not resulted inhibited and the up-regulation of UCP2 (only in 250 μ M
412 La+Ce group) was recorded. This demonstrate the different behaviour of Colon adenocarcinoma
413 cell lines compared to hepatocellular carcinoma lines, also if they similarly shown a basal UCP2
414 overexpression (Derdak et al., 2008; Santandreu et al., 2009).

415 Super Oxide Dismutases (SOD) are a family of evolutionarily conserved enzymes that dismutate
416 superoxide free radicals: SOD2 isoform is localized in the mitochondrial matrix, in association with
417 mtDNA (Xu et al., 2008). Reduced SOD2 mRNA is strongly associated with poor survival in HCC
418 patients, correlated with larger tumor size, multiple tumor nodules and tumor emboli, and cancer

recurrence (Wang et al., 2016). Increased expression levels of SOD2 was recorded in HEPG2 stimulated for 48 h with La 180 μ M, La+Ce 100 μ M and La+Ce 250 μ M but its overexpression doesn't seem to prevent the strong inhibition of HEPG2 proliferation found performing MTT tests (Table 1). The controversial effects on oxidative stress response genes during the performed REE stimulation protocols need to be further investigated in order to understand if activated ROS pathways found in present study are exclusively related to specific cancer phenotype of used cell lines, or could be extended to normal cells.

In conclusion, the expression profiles of certain genes in La and Ce treated cells differed markedly from those of control cell lines. This is consistent with the wide biological effect spectrum of REE elements. Multiple genes may act together to play a same role, and further analysis of the differentially expressed genes would be helpful for understanding the wide biological effect spectrum of REE elements.

Acknowledgements

This work was supported by the Italian Ministry of Health [Ricerca Corrente 2012, Grant number 22RC12]

Figure captions

Figure 1: Effect of lanthanides on cell viability

HepG2 (panel A) and HT-29 cells (panel B) were plated (10×10^4 cells/well) in a 96-well plate, treated with the test compounds for the indicated times and then incubated for 10 min at 37°C with RealTime-Glo™ reagent. Luminescence was measured with an integration time of 0.5 second per well on a Glomax Multi Detection System Promega. The fold change in cell viability of lanthanides-treated cells was compared to control cells. Data are expressed as mean of luminescence values (RLU) \pm SD (Standard Deviation) of 2 experiments conducted in 6 replicates. *= p<0.05; **= p<0.01; ***=p<0.001.

Figure 2: Effect of lanthanides on Proliferating Cell Nuclear Antigen (PCNA) expression.

448 Western Blotting analysis of PCNA performed on total extracts of HepG2 (panel **A**) and HT-29
449 (panel **B**) cells treated or not with different concentration of Lanthanides for indicating time. Equal
450 loading was evaluated by re-probing membranes with β -actin. Densitometry values are indicated.

451

452 **Figure 2A**

453 HT-29 - Genes differentially expressed at 48 hours of treatment (all groups with at least one of the
454 treatments with $-2 < \Delta Ct < 2$)

455

456 **Figure 2B**

457 HT-29 - Genes differentially expressed at 48 hours of treatment (only groups with $-2 < \Delta Ct < 2$)

458

459 **Figure 3A**

460 HepG2 - Genes differentially expressed at 48 hours of treatment (all groups with at least one of the
461 treatments with $-2 < \Delta Ct < 2$)

462

463 **Figure 3B**

464 HepG2 - Genes differentially expressed at 48 hours of treatment (only groups with $-2 < \Delta Ct < 2$)

465

466

467

468

469

References

- Arvela, P., Kraul, H., Stenback, F., Pelkonen, O., 1991. The cerium-induced liver injury and oxidative drug metabolism in DBA/2 and C57BL/6 mice. *Toxicology* 69, 1-9.
- Barashi, N., Weiss, I.D., Wald, O., Wald, H., Beider, K., Abraham, M., Klein, S., Goldenberg, D., Axelrod, J., Pikarsky, E., Abramovitch, R., Zeira, E., Galun, E., Peled, A., 2013. Inflammation-induced hepatocellular carcinoma is dependent on CCR5 in mice. *Hepatology* 58, 1021-1030.
- Bicker, A., Dietrich, D., Gleixner, E., Kristiansen, G., Gorr, T.A., Hankeln, T., 2014. Extensive transcriptional complexity during hypoxia-regulated expression of the myoglobin gene in cancer. *Hum Mol Genet* 23, 479-490.
- Cannito, S., Novo, E., Compagnone, A., Valfrè di Bonzo, L., Busletta, C., Zamara, E., Paternostro, C., Povero, D., Bandino, A., Bozzo, F., Cravanzola, C., Bravoco, V., Colombatto, S., Parola, M., 2008. Redox mechanisms switch on hypoxia-dependent epithelial-mesenchymal transition in cancer cells. *Carcinogenesis* 29, 2267-2278.
- Chakraborty, S., John, R., Nag, A., 2014. Cytochrome in tumor hypoxia: novel insights into cancer suppression. *Tumour Biol* 35, 6207-6219.
- Chen, A., Chen, D., Liu, Y., Liu, P., Wang, X., Nie, Y., Wang, X., Sun, S., 2000. The long term effect of low dose of Changle on the structure and function of liver in rats. *Zhonghua Yufang Yixue* 34, 46-48.
- Dai, Y., Li, J., Yu, L., Dai, G., Hu, A., Yuan, L., Wen, Z., 2002. Effects of rare earth compounds on growth and apoptosis of leukemic cell lines. *In Vitro Cell Dev Biol Anim* 38, 373-375.
- Derdak, Z., Mark, N.M., Beldi, G., Robson, S.C., Wands, J.R., Baffy, G., 2008. The mitochondrial uncoupling protein-2 promotes chemoresistance in cancer cells. *Cancer Res* 68, 2813-2819.
- Drew, L.J., Qingrun, M., Weijun, S., 1990. The Bayan Obo iron rare-earth niobium deposits, Inner-Mongolia, China. *Lithos* 26, 43-65.
- EFSA, 2013. Scientific Opinion on the safety and efficacy of Lancer (lanthanide citrate) as feed additive for weaned piglets. *EFSA Journal* 11, 3206.
- Guariniello, S., Di Bernardo, G., Colonna, G., Cammarota, M., Castello, G., Costantini, S., 2015. Evaluation of the selenotranscriptome expression in two hepatocellular carcinoma cell lines. *Anal Cell Pathol (Amst)* 2015, 419561.
- Guerriero, E., Capone, F., Accardo, M., Sorice, A., Costantini, M., Colonna, G., Castello, G., Costantini, S., 2015. GPX4 and GPX7 over-expression in human hepatocellular carcinoma tissues. *Eur J Histochem* 59, 2540.
- He, M.L., Rambeck, W.A., 2000. Rare earth elements: a new generation of growth promoters for pigs? *Arch Tierernahr* 53, 323-334.
- He, M.L., Wang, Y.Z., Xu, Z.R., Chen, M.L., Rambeck, W.A., 2003. Effect of dietary rare earth elements on growth performance and blood parameters of rats. *J Anim Physiol Anim Nutr (Berl)* 87, 229-235.
- He, R., Xia, Z., 1998. Effects of rare earth elements on growing and fattening of pigs. *Guangxi Agric Sci* 5, 243-245.
- Helbo, S., Weber, R.E., Fago, A., 2013. Expression patterns and adaptive functional diversity of vertebrate myoglobins. *Biochim Biophys Acta* 1834, 1832-1839.
- Huang, P., Li, J., Zhang, S., Chen, C., Han, Y., Liu, N., Xiao, Y., Wang, H., Zhang, M., Yu, Q., Liu, Y., Wang, W., 2011. Effects of lanthanum, cerium, and neodymium on the nuclei and mitochondria of hepatocytes: accumulation and oxidative damage. *Environ Toxicol Pharmacol* 31, 25-32.
- Khan, N., 2004. Alternatives to in-feed antibiotics. *Feed Magazine* 7-8, 236-239.
- Li, H., Cao, Z., Zhang, G., Thannickal, V.J., Cheng, G., 2012. Vascular peroxidase 1 catalyzes the formation of hypohalous acids: characterization of its substrate specificity and enzymatic properties. *Free Radic Biol Med* 53, 1954-1959.

Li, X.Q., Jiang, J.J., Yang, L., Liu, M., Yang, L.Y., 2000. Effect of low level mixed rare earth Changle on learning and memory, locomotor activity and NMDA-and M-receptors activity and morphology of divisions of hippocampal cortex in rats. *Zhonghua Yufang Yixue Zazhi* 34, 20-23.

Liu, J.M., Chen, D., Wang, X.M., Nie, Y.X., Li, Y., Li, J., 2000. Long-term effect of Changle and $\text{La}(\text{NO}_3)_3$ on level of IL-2 and g-IFN of splenic lymphocytes in rats. *Zhonghua Yufang Yixue Zazhi* 34, 49-51.

Liu, X., Tong, J., Zweier, J.R., Follmer, D., Hemann, C., Ismail, R.S., Zweier, J.L., 2013. Differences in oxygen-dependent nitric oxide metabolism by cytoglobin and myoglobin account for their differing functional roles. *FEBS J* 280, 3621-3631.

Martin, D., Rojo, A.I., Salinas, M., Diaz, R., Gallardo, G., Alam, J., De Galarreta, C.M., Cuadrado, A., 2004. Regulation of heme oxygenase-1 expression through the phosphatidylinositol 3-kinase/Akt pathway and the Nrf2 transcription factor in response to the antioxidant phytochemical carnosol. *J Biol Chem* 279, 8919-8929.

Mosmann, T., 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J Immunol Methods* 65, 55-63.

Nakamura, Y., Tsumura, Y., Tonogai, Y., Shibata, T., Ito, Y., 1997. Differences in behavior among the chlorides of seven rare earth elements administered intravenously to rats. *Fundam Appl Toxicol* 37, 106-116.

Pyo, M.C., Yang, S.Y., Chun, S.H., Oh, N.S., Lee, K.W., 2016. Protective Effects of Maillard Reaction Products of Whey Protein Concentrate against Oxidative Stress through an Nrf2-Dependent Pathway in HepG2 Cells. *Biol Pharm Bull* 39, 1437-1447.

Rambeck, W.A., He, M.L., Wehr, U., 2004. Influence of the alternative growth promoter "Rare Earth Elements" on meat quality in pigs., *Proceedings International Conference pig and poultry meat safety and quality of meat science and technology*, Krakow, Poland.

Rambeck, W.A., Wehr, U., 2000. Rare earth elements as alternative growth promoters in pig production. *Arch Tierernahr* 53, 323-334.

Redling, K., 2006. Rare earth elements in agriculture with emphasis on animal husbandry. Ludwig-Maximilians-Universität, München.

Rouimi, P., Zucchini-Pascal, N., Dupont, G., Razpotnik, A., Fouche, E., De Sousa, G., Rahmani, R., 2012. Impacts of low doses of pesticide mixtures on liver cell defence systems. *Toxicol In Vitro* 26, 718-726.

Salminen, A., Kaarniranta, K., Haapasalo, A., Hiltunen, M., Soininen, H., Alafuzoff, I., 2012. Emerging role of p62/sequestosome-1 in the pathogenesis of Alzheimer's disease. *Prog Neurobiol* 96, 87-95.

Santandreu, F.M., Valle, A., Fernandez de Mattos, S., Roca, P., Oliver, J., 2009. Hydrogen peroxide regulates the mitochondrial content of uncoupling protein 5 in colon cancer cells. *Cell Physiol Biochem* 24, 379-390.

Schwabe, A., Meyer, U., Flachowsky, G., Danicke, S., 2011. Effect of graded levels of rare earth elements in diets of fattening bulls on growing and slaughtering performance, and on nutrient digestibility of wethers. *Arch Anim Nutr* 65, 55-73.

Shah, P., Djisam, R., Damulira, H., Aganze, A., Danquah, M., 2016. Embelin inhibits proliferation, induces apoptosis and alters gene expression profiles in breast cancer cells. *Pharmacol Rep* 68, 638-644.

Soudi, M., Paumann-Page, M., Delporte, C., Pirker, K.F., Bellei, M., Edenhofer, E., Stadlmayr, G., Battistuzzi, G., Boudjeltia, K.Z., Furtmuller, P.G., Van Antwerpen, P., Obinger, C., 2015. Multidomain human peroxidase 1 is a highly glycosylated and stable homotrimeric high spin ferric peroxidase. *J Biol Chem* 290, 10876-10890.

Valdes, A., Garcia-Canas, V., Rocamora-Reverte, L., Gomez-Martinez, A., Ferragut, J.A., Cifuentes, A., 2013. Effect of rosemary polyphenols on human colon cancer cells: transcriptomic profiling and functional enrichment analysis. *Genes Nutr* 8, 43-60.

570 Valle, A., Oliver, J., Roca, P., 2010. Role of uncoupling proteins in cancer. *Cancers (Basel)* 2, 567-
571 591.

572 Von Rosenberg, S., Rambeck, W., Wehr, U., 2013. Lanthanum and cerium do not accumulate in
573 organs, muscle or bone following highly elevated oral supplementation of lanthanide citrate in
574 weaned piglets. , *Proceedings of the Society of Nutrition and Physiology*, p. 183.

575 Wang, M.Q., Xu, Z.R., 2003. Effect of supplemental lanthanum on the growth performance of pigs.
576 *Asian-Aust. J. Anim. Sci.* 16, 1360-1363.

577 Wang, R., Yin, C., Li, X.-X., Yang, X.-Z., Yang, Y., Zhang, M.-Y., Wang, H.-Y., Zheng, X.F.S.,
578 2016. Reduced SOD2 expression is associated with mortality of hepatocellular carcinoma patients
579 in a mutant p53-dependent manner. *Aging (Albany NY)* 8, 1184-1197.

580 Wang, S.C., 2014. PCNA: a silent housekeeper or a potential therapeutic target?. *Trends Pharmacol*
581 *Sci.* 35, 178-86

582 Xu, H.E., Gao, G.H., Jia, F.L., Wang, X.Y., Xie, Q., Liu, H.S., Wang, N.F., 2000. Effect of mixed
583 rare earth Changle on the micronuclei formation of blood lymphocytes in rats. . *Zhonghua Yufang*
584 *Yixue Zazhi* 34, 5-7.

585 Xu, Y., Fang, F., Dhar, S.K., Bosch, A., St Clair, W.H., Kasarskis, E.J., St Clair, D.K., 2008.
586 Mutations in the SOD2 promoter reveal a molecular basis for an activating protein 2-dependent
587 dysregulation of manganese superoxide dismutase expression in cancer cells. *Mol Cancer Res* 6,
588 1881-1893.

589 Xue, L., Ping, H., Jie, X., Shiwei, S., Jinhai, L., L., Y., 2009. Effect of lanthanum chloride on
590 growth of breast cancer cells and regulation of c-met transcription. *Frontiers of Medicine in China*
591 3, 336-340.

592 Yang, Z.M., A., W., 2006. Carbonatites in China: a review. *Journal of Asian Earth Sciences* 27,
593 559-575.

594 Yu, G., Liu, J., Xu, K., Dong, J., 2015a. Uncoupling protein 2 mediates resistance to gemcitabine-
595 induced apoptosis in hepatocellular carcinoma cell lines. *Biosci Rep* 35.

596 Yu, L., Xiong, J., Guo, L., Miao, L., Liu, S., Guo, F., 2015b. The effects of lanthanum chloride on
597 proliferation and apoptosis of cervical cancer cells: involvement of let-7a and miR-34a microRNAs.
598 *Biometals* 28, 879-890.

599 Zhao, H., Hao, W.D., Xu, H.E., Shang, L.Q., Lu, Y.Y., 2004. Gene expression profiles of
600 hepatocytes treated with La(NO₃)₃ of rare earth in rats. *World J Gastroenterol* 10, 1625-1629.

601 Zhu, X., Li, D., Yang, W., Xiao, C., Chen, H., 1994. Effects of rare earth elements on the growth
602 and nitrogen balance of piglets. . *Feed Industry* 15, 23-25.

603

604